Bile canaliculi formation by aligning rat primary hepatocytes in a microfluidic device

Yosuke Nakao,[1] Hiroshi Kimura,[1,2] Yasuyuki Sakai,[1,2] and Teruo Fujii[1,2,a)]
[1]*Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan*
[2]*Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Tokyo, Japan*

(Received 13 December 2010; accepted 29 March 2011; published online 29 June 2011)

In this study, we propose a microfluidic cell culture device mimicking the microscopic structure in liver tissue called hepatic cords. The cell culture area of the device was designed to align hepatocytes in two lines in a similar way to hepatic cords. Thanks to the structural design together with a cell seeding procedure, rat primary hepatocytes were successfully aligned in two lines and cultured under perfusion condition. It is shown that aligned hepatocytes gradually self-organize and form bile canaliculi along the hepatic cord-like structure. The present technique to culture hepatocytes with functional bile canaliculi could be used as an alternative to animal testing in the field of drug discovery and toxicological studies, and also be beneficial to tissue engineering applications. © *2011 American Institute of Physics.* [doi:10.1063/1.3580753]

I. INTRODUCTION

The liver, which is one of the vital organs to maintain physiological homeostasis, has a variety of significant biological functions. To reconstruct functional liver tissue *in vitro* using hepatocytes is enormously beneficial in the fields of toxicity assessment, pharmaceutical development, and tissue engineering.[1–4] For a physiologically relevant model, to obtain hepatocytes with membrane polarity and functional bile canaliculi is essential.[5] However, freshly isolated hepatocytes rapidly lose their membrane polarity. This causes a decline in liver-specific functions.[6,7]

One of the most effective techniques to solve this problem is a collagen sandwich culture. By this method, hepatic functions can be maintained *in vitro* for a long term.[8,9] Another valuable technique is to form spheroids, which also induce the hepatic activity.[10,11]

Emerging microfabrication and microfluidic technologies also have a potential to improve cell culture systems, enabling the control of the cellular microenvironment at the microscale.[12] It was shown that perfusion culture mimicking continuous blood flow induced the liver-specific functions.[13] Lee *et al.* developed an artificial liver sinusoid with an endothelial-like barrier mimicking liver tissue. In their culture device, hepatocytes can be cultured under perfusion condition without shear stress.[14] Moreover, microfluidics can contribute to the development of three-dimensional culture systems.[15,16] These microfluidic culture technologies have been applied, for example, to drug hepatotoxicity testing.[17,18]

One challenging problem of current liver tissue culture is lack of a separate compartment to collect secreted bile.[19] In native livers, the secreted bile from hepatocytes is excreted in bile canaliculi and discharged into bile ducts. On the other hand, current culture methods have no excretion system and the bile is accumulated in the cultured hepatocytes. This shows toxicity to

a)Author to whom correspondence should be addressed. Tel.: +81-3-5452-6211. FAX: +81-3-5452-6212. Electronic mail: tfujii@iis.u-tokyo.ac.jp.

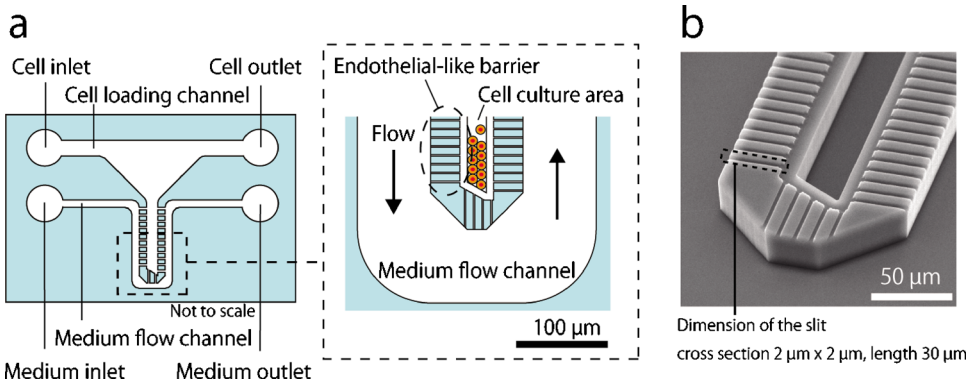

FIG. 1. (a) Design of a microfluidic device mimicking the structure of a hepatic cord. The device has four terminal ports and consists of flow channels, a cell culture area, and an endothelial-like barrier. The width of the cell culture area is designed to be 37 μm, which enables to align hepatocytes in two lines. Culture medium is perfused in the flow channel. (b) An image of the device structure by scanning electron microscope.

hepatocytes.[20] One limitation preventing ones from solving this problem is that the formation of bile canaliculi cannot be controlled because they are formed at random locations in the conventional culture methods.

In this study, we fabricated a microfluidic cell culture device mimicking the structure of liver tissue following the work done by Lee *et al.*[14] The design of the cell culture area was modified to be an asymmetrical shape with an optimized dimension to align the inoculated cells into two lines. Perfusion culture of the cells aligned in the device was conducted to form bile canaliculi along this hepatic cord-like structure.

II. MATERIALS AND METHODS

A. Design of microfluidic device

A microfluidic device was designed to mimic *in vivo* environment and the structure of hepatic cords (Fig. 1). The device consists of a medium flow channel (100 μm width and 30 μm height), a cell loading channel (200 μm width and 30 μm height), a cell culture area (37 μm width and 30 μm height), and an endothelial-like barrier.[14] In order to align hepatocytes smoothly in two lines, the tip of the cell culture area is designed to be asymmetrical, and its width is 37 μm to accommodate two cells side-by-side. The endothelial-like structure, previously reported by Lee *et al.*,[14] is a wall-like structure surrounding the cell culture area to avoid the cells to be exposed directly to the culture medium flow. A series of 30 μm long narrow slits are formed on top of the wall with 10 μm intervals to allow nutrition to go into the cell culture area. In order to minimize the cross sectional area of the slits to avoid deformation of the cells, the dimension of the cross section was determined to be 2 μm wide and 2 μm high considering the lower size limit of fabrication.

To confirm the functions of the device, COMSOL MULTIPHYSICS (COMSOL, Inc.) was used for the simulation of the flow in the device (Fig. 2). The simulation results show that the flow velocity at the medium flow channel is 1 mm/s, which is close to the velocity of blood flow *in vivo*. The flow velocity in the cell culture area is 0 mm/s under perfusion condition (flow rate is 0.1 μl/min, and both cell inlet and cell outlet are closed) due to much greater fluidic resistance of the endothelial-like barrier than that of the medium flow channel. It is also shown that nutrients could be supplied to the cells by diffusion.

B. Fabrication

The microfluidic device was fabricated by conventional photolithography and soft lithography techniques.[21–23] A mold master was fabricated as in the following. After the cleaning step, an

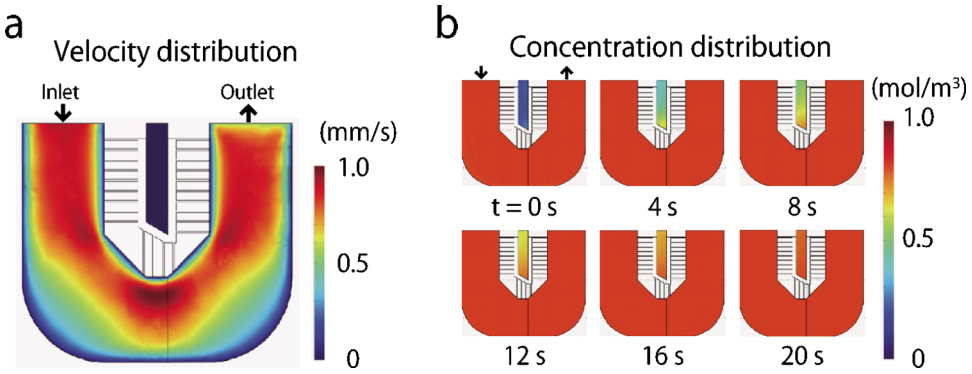

FIG. 2. Results of the numerical simulation. (a) Velocity distribution. The velocity in the cell culture area was 0 mm/s, even when medium is perfused in the flow channel. (b) Concentration distribution. Substances gradually diffused into the cell culture area and the concentration became almost homogeneous in 20 s.

ultrathick photoresist SU-8 2002 (MICRO CHEM) was spin-coated on a 4 in. silicon wafer to achieve a thick layer of 2 μm high. The SU-8 layer was exposed to UV light for several seconds and baked. To make multiple layers, an additional layer of SU-8 2035 (MICRO CHEM) was spin-coated on the top of the first layer. After the alignment of first and second layers by a mask aligner (PEM-800, Union), SU-8 layers were exposed and developed. The SU-8 mold master was coated with CHF_3 for passivation. The complete SU-8 mold was used for PDMS (polydimethylsiloxane, Silpot 184, DowCorning) replica molding. After peeled off from the mold master, the PDMS layer was permanently bonded to a slide glass through oxygen plasma activation [O_2, 50 SCCM (SCCM denotes cubic centimeter per minute at STP), 20 Pa, 75 W, 10 s, RIE-10NR, SAMCO]. The fabricated device was connected to disposable syringes with Teflon™ tubes (inner diameter is 1 mm).

C. Characterization of the device

The flow velocity and the substance diffusion in the microfluidic device were evaluated by the following methods. Fluorescent beads suspension (0.4 μm diameter, 0.1% v/v) in PBS (phosphate buffered saline) was used for the evaluation of the flow velocity. After all the channels were filled with the beads suspension, additional suspension was injected through the medium flow channel by a syringe pump at the flow rate of 0.1 μl/min. For the evaluation of substance diffusion, fluorescein in PBS solution (100 μM) was used. After the channel was filled with PBS without fluorescein, the fluorescein solution was injected through a medium flow channel by a syringe pump at the flow rate of 0.1 μl/min. The motion of the fluorescent beads and fluorescence intensity were observed using a fluorescence microscope (PowerIX71, OLYMPUS).

D. Isolation of rat hepatocytes

Hepatocytes were isolated from a rat liver by a collagenase perfusion method with some modifications.[24] Male Wister rats (Sankyo Laboratory Service, Tokyo, Japan) weighing 200–300 g were used. All of the study protocols were reviewed and approved by the Animal Ethics Committee of the Institute of Industrial Sciences at the University of Tokyo. Cell pellets were resuspended in a culture medium [high-glucose Dulbecco's Modified Eagle's Medium (Invitrogen) with the addition of 10% fetal bovine serum, 1 μM dexamethasone (Wako), 0.1 μM insulin (Takara), 10ng/ml EGF (Epidermal Growth Factor, Wako), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen)]. Cells' viability was determined by a trypan blue exclusion method. Cells with greater than 85% viability were used for further studies.

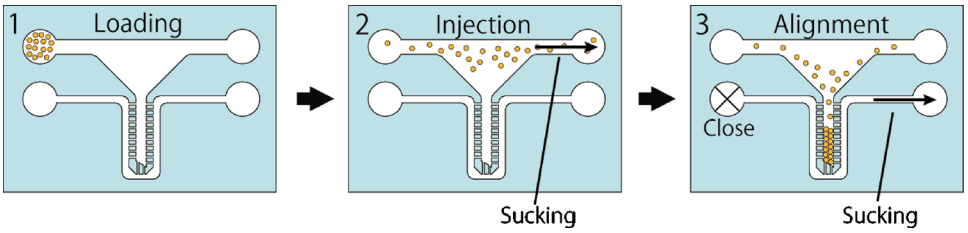

FIG. 3. Procedure of aligning cells in the microfluidic device. Rat primary hepatocytes were loaded via the cell inlet and injected in the cell loading channel by applying negative pressure at the cell outlet. Then, hepatocytes were pulled and aligned in two lines by applying negative pressure at the medium outlet.

E. Cell alignment and culture in the device

The inner walls of the microfluidic device were coated with collagen (Cellmatrix Type 1-P, NITTA GELATIN) by injecting 0.3% collagen solution. After incubation for 1 h, the channels were rinsed with PBS and filled with culture medium. Cells were aligned in the microfluidic device by the following procedure (Fig. 3). First, cell suspension (1×10^7 cells/ml) was loaded from the cell inlet and injected into the cell loading channel by sucking with a syringe from cell outlet. Next, cells were pulled into the cell culture area by sucking with a syringe from the medium outlet. Thanks to the higher fluidic resistance of the endothelial-like barrier, cells can softly be introduced into the cell culture area and aligned properly by the sucking process. It was observed that the moving velocity of the cells during this process is approximately 50 μm/s, which caused no damage to the cells. After the cell seeding procedure, cell inlet and cell outlet were closed. Then, the alignment of the cells was checked under a microscope (PowerIX71, OLYMPUS). The device containing the cells successfully aligned in two lines was incubated for 1 h with no flow for cell attachment. After these processes, culture medium with 150 μg/ml Matrigel (BD Matrigel, BD) was perfused by a syringe pump at the flow rate of 0.1 μl/min. Matrigel was added to promote reconstruction of bile canaliculi.[25] As a control experiment, cells were cultured on a glass-bottom 24 well plate (EZVIEW). Cells were seeded at the density of 1.2×10^5 cells/cm^2. Culture medium was changed every day. All cells were cultured in an incubator (5% CO_2, 37 °C).

F. Immunostaining

The cells were fixed with 4% paraformaldehyde in PBS for 15 min and then were blocked with 0.05% Triton X-100 and 1% bovine serum albumin in PBS at room temperature for 1 h. Next, the cells were incubated with a primary mouse anti-MRP2 monoclonal antibody (Enzo), a mouse anti-CD147 monoclonal antibody (AbD Serotec), and a rabbit ZO-1 polyclonal antibody (Invitrogen) for 12 h at room temperature. After rinses with PBS, the cells were incubated with secondary Alexa Fluor 546 anti-mouse IgG (Invitrogen) or Alexa Fluor 488 anti-rabbit IgG (Invitrogen) at room temperature for 1 h. The cells were rinsed with PBS, counterstained with Hoechst 33342, placed in fluorescent mounting medium (Dako, Denmark), and observed by a fluorescence microscope (PowerIX71, OLYMPUS).

G. Analysis of bile canaliculi formation and function

The bile canaliculi formation and function was verified by using the substrate of MRP2, 5-(and-6)-carboxy-2′,7′-dichloro-fluorescein (CDF). 5 μM 5-(and-6)-carboxy-2′,7′-dichloro-fluorescein diacetate (CDFDA, Invitrogen) solution in PBS (Ca+,Mg+) was injected into the device. CDFDA was absorbed by the cells and metabolized into CDF by esterase. The metabolites were actively excreted into bile canaliculi by MRP2 protein.[26] After 20 min incubation, the cells were rinsed with PBS (Ca+,Mg+). The morphology of hepatocytes and the accumulation of CDF in the bile canaliculi were observed by a fluorescence microscope (PowerIX71, OLYMPUS).

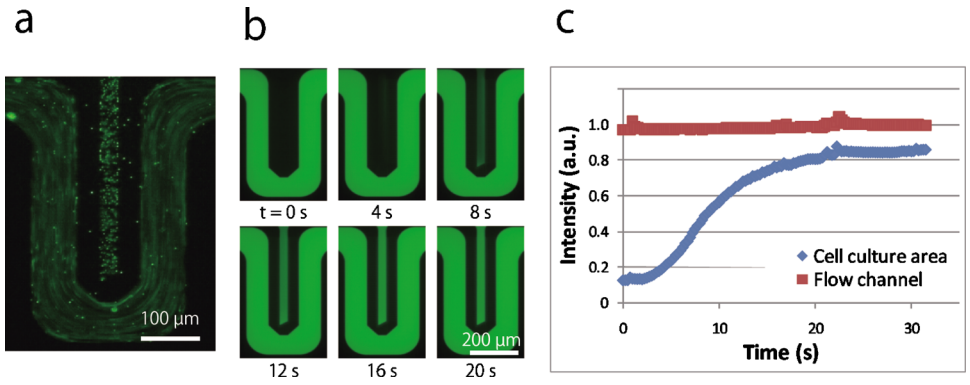

FIG. 4. Results of the device characterization. (a) Fluorescence beads suspension was injected into the flow channel at a flow rate of 0.1 μl/min. While the measured flow velocity in the flow channel was calculated to be 1.36 mm/s, no flow was observed in the cell culture area. (b) The fluorescein solution (100 μM in PBS) was injected into the medium flow channel at a flow rate of 0.1 μl/min. The fluorescein gradually diffused into the cell culture area. (c) Measurement of fluorescence intensity at a location in the flow channel and in the cell culture area. These results show good agreement with the simulation results shown in Fig. 3.

III. RESULTS AND DISCUSSION

A. Characterizations of the device

The flow velocity in the device was evaluated by capturing the motion of the fluorescent beads in the channel as a movie. The maximum velocity at the center of the medium flow channel calculated from the movie was 1.36 mm/s, while no flow was observed in the cell culture area [Fig. 4(a)]. The calculated shear stress value from the flow velocities at the medium flow channel is 1.3 Pa, while no shear stress in the cell culture area. Although it is known that cells exposed to high shear rates show reduction of liver-specific functions,[27] this result shows that cells could be cultured under perfusion condition without shear stress. For the evaluation of substance diffusion, fluorescence intensity was measured. The fluorescein gradually diffused from the medium flow channel to the cell culture area through the endothelial-like barrier [Fig. 4(b)]. This result shows that substances could be supplied to the cells continuously by diffusion under perfusion condition. The concentration of the cell culture area was almost saturated after 20 s, showing good agreement with the simulation result. This result shows that cells can obtain nutrients from perfused culture medium continuously. Figure 4(c) shows the concentration was almost saturated after 20 s, but the concentration of the cell culture area did not correspond to that of the medium flow channel. This is because the molecules diffused into the cell loading channel, of which the volume was much greater than that of the cell culture area.

B. Hepatocyte culture in the microfluidic device

Rat primary hepatocytes were successfully aligned in two lines by the geometric design of the cell culture area and the cell seeding procedure without visible damages [Fig. 6(a)]. Seeded cells adhered well enough on the cell culture area after 1 h incubation with no flow. Then, the cells gradually self-organized and formed cell-cell interaction as the perfusion culture proceeded. Cells were successfully cultured for 4 days and immunostaining and CDFDA assay were done at day 4.

To verify the extent of repolarization in hepatocytes culture in the device, we examined the expression of typical markers for the apical and basolateral membrane sides by immunostaining. The results of immunostaining are shown in Fig. 5. The apical marker MRP2 was concentrated in the localized regions along the cell-cell contacts and the basolateral marker CD147 protein was expressed in almost all the areas across the channel corresponding to the locations of cell membranes. These distributions of the marker proteins were similar to those of *in vivo*. This means that cultured hepatocytes were repolarized.

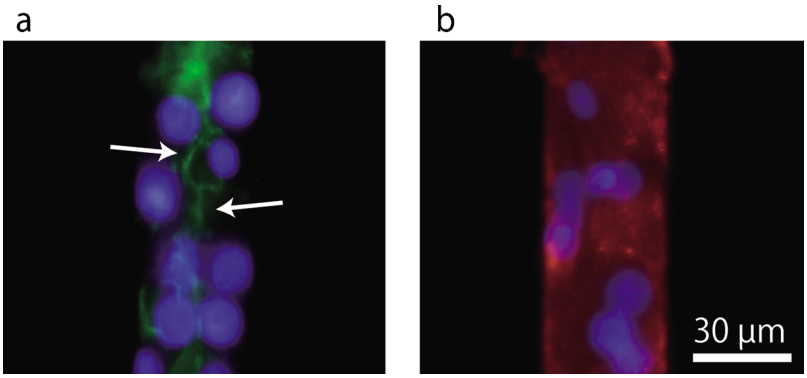

FIG. 5. Results of the immunostaining. (a) The distribution of apical marker MRP2 protein (green fluorescence). MRP2 was concentrated in the localized regions along the cell-cell contacts. (b) The distribution of basolateral marker CD147 protein (red fluorescence). CD147 was expressed in almost all the areas across the channel corresponding to the locations of cell membranes.

The result of CDFDA assay in the microfluidic device is shown in Fig. 6(b). CDFDA is absorbed by cells and metabolized into CDF (fluorescent metabolite). CDF is actively excreted into bile canaliculi by MRP2 protein, which is an important transporter protein that modulates pharmacokinetics of many drugs. In Fig. 6(b), it is shown that fluorescent substrates were accumulated between the aligned cells, where cells formed bile canaliculi with tight junction. This result indicates that cells were repolarized through cell culture, and had metabolic activity and MRP2 transport function. These functional activities may be enhanced by the culture medium perfusion, which can transport nutrients and waste products continuously.[16] Furthermore, hepatocytes cultured in the microfluidic device formed bile canaliculi in a linear shape along the hepatic cord-like structure. On the contrary, formation of bile canaliculi on a well plate was at random [Fig. 6(c)]. This result indicates that formation of bile canaliculi can be controlled by aligning the cells in two lines in the present hepatic cord-like structure.

The hepatocyte culture system with separated compartments of a bile canaliculi area and a sinusoid area can be a useful model for the analyses of drug metabolism and excretion. And the controlled formation of bile canaliculi has a potential to make it possible to collect bile. Continuous bile collection will help hepatocytes live longer stably with liver-specific functions.

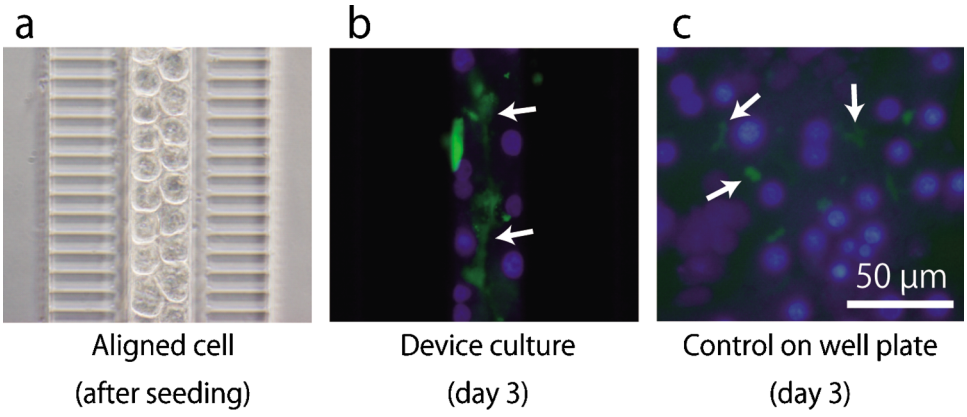

FIG. 6. Results of cell alignment and culture. (a) Aligned cells in two lines like a hepatic cord. (b) Bile canaliculi formation in the cell culture area. Hepatocytes formed bile canaliculi along the hepatic cord-like structure. (c) Control experiment in a 24 well plate. Bile canaliculi were formed randomly in the well plate.

IV. CONCLUSION

In this study, we newly designed a microfluidic cell culture device with a hepatic cord-like structure. Thanks to the optimized shape and dimension of the cell culture area and the cell seeding procedure, the hepatocytes could be aligned in two lines mimicking the arrangement of the cells in the hepatic cord *in vivo*. It is confirmed that the hepatocytes in the device could be cultured under perfusion condition without shear stress. Hepatocytes aligned in the device formed bile canaliculi in a linear shape along the hepatic cord-like structure. The device can be used as an *in vitro* model for the analyses of metabolism and excretion, and could be one of the useful alternatives to animal testing. The cultured hepatocytes with functional bile canaliculi could also be valuable for future liver tissue engineering.

ACKNOWLEDGMENTS

This work was supported by the Bio Electromechanical Autonomous Nano Systems (BEANS) Project.

[1] K. Behnia, S. Bhatia, N. Jastromb, U. Balis, S. Sullivan, M. Yarmush, and M. Toner, Tissue Eng. **6**, 467 (2000).
[2] I. Grattagliano, P. Portincasa, V. O. Palmieri, and G. Palasciano, Annals of Hepatology **1**, 162 (2002).
[3] W. Hashimoto, R. Sudo, K. Fukasawa, M. Ikeda, T. Mitaka, and K. Tanishita, Pathology **173**, 494 (2008).
[4] B. E. Uygun, A. Soto-Gutierrez, H. Yagi, M. L. Izamis, M. A. Guzzardi, C. Shulman, J. Milwid, N. Kobayashi, A. Tilles, F. Berthiaume, M. Hertl, Y. Nahmias, M. L. Yarmush, and K. Uygun, Nat. Med. **16**, 814 (2010).
[5] M. A. Talamini, B. Kappus, and A. Hubbard, Hepatology (Philadelphia, PA, U. S.) **25**, 167 (1997).
[6] C. Rodríguez-Antona, M. T. Donato, A. Boobis, R. J. Edwards, P. S. Watts, J. V. Castell, and M. J. Gómez-Lechón, Xenobiotica **32**, 505 (2002).
[7] F. Boess, M. Kamber, S. Romer, R. Gasser, D. Muller, S. Albertini, and L. Suter, Toxicol. Sci. **73**, 386 (2003).
[8] C. Y. Dunn, R. G. Tompkins, and M. L. Yarmush, Biotechnol. Prog. **7**, 237 (1991).
[9] F. Berthiaume, P. V. Moghe, M. Toner, and M. L. Yarmush, FASEB J. **10**, 1471 (1996).
[10] J. Landry, D. Bernier, C. Ouellet, R. Goyette, and N. Marceau, J. Cell Biol. **101**, 914 (1985).
[11] S. F. Abu-Absi, J. R. Frienda, L. K. Hansenb, and W. S. Hu, Exp. Cell Res. **274**, 56 (2002).
[12] H. Andersson and A. V. Berg, Sens. Actuators B **92**, 315 (2003).
[13] E. Leclerc, Y. Sakai, and T. Fujii, Biotechnol. Prog. **20**, 750 (2004).
[14] P. J. Lee, P. J. Hung, and L. P. Lee, Biotechnol. Bioeng. **97**, 5 (2007).
[15] Y. C. Toh, C. Zhang, J. Zhang, Y. M. Khong, S. Chang, V. D. Samper, D. V. Noort, D. W. Hutmacher, and H. Yu, Lab Chip **7**, 302 (2007).
[16] V. N. Goral, Y. C. Hsieh, O. N. Petzold, J. S. Clark, P. K. Yuen, and R. A. Faris, Lab Chip **10**, 3380 (2010).
[17] L. Xia, S. Ng, R. Han, X. Tuo, G. Xiao, and H. L. Leo, Biomaterials **30**, 5927 (2009).
[18] E. Novik, T. J. Maguire, P. Chao, K. C. Cheng, and M. L. Yarmush, Biochem. Pharmacol. **79**, 1036 (2010).
[19] Y. Nahmias, F. Berthiaume, and M. L. Yarmush, Adv. Biochem. Eng./Biotechnol. **103**, 309 (2006).
[20] P. Ujhazy, D. Ortiz, S. Misra, S. Li, J. Moseley, H. Jones, and I. M. Arias, Hepatology (Philadelphia, PA, U. S.) **34**, 768 (2001).
[21] T. Fujii, Microelectron. Eng. **61–62**, 907 (2002).
[22] Y. Xia and G. M. Whitesides, Angew. Chem. **37**, 1433 (1998).
[23] D. C. Duffy, J. C. McDonald, O. J. A. Schueller, and G. M. Whitesides, Anal. Chem. **70**, 4974 (1998).
[24] P. O. Seglen, Methods Cell Biol. **13**, 29 (1976).
[25] D. M. Bissell, D. M. Arenson, J. J. Maher, and F. J. Roll, J. Clin. Invest. **79**, 801 (1987).
[26] X. Tian, M. J. Zamek-Gliszczynski, P. Zhang, and K. L. Brouwer, Mol. Pharmacol. **66**, 1004 (2004).
[27] A. W. Tilles, H. Baskaran, P. Roy, M. L. Yarmush, and M. Toner, Biotechnol. Bioeng. **73**, 379 (2001).